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Protective Effect of Oral Ascorbic Acid (Vitamin C) Against Acetaminophen-Induced Hepatic Injury in Rats

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ABSTRACT

The incidence of acetaminophen-induced hepatotoxicity is reported to be on the increase, with limited therapeutic or chemoprophylactic options. In the present in vivo study, single daily oral doses (100 – 500 mg/kg) of ascorbic acid (ASC) were investigated for their protective effects against acetaminophen (APAP)-induced hepatotoxicity in 4 groups of rats made up of 6 rats per group for 14 days. Also, effects of the doses on body weights taken on the 1st, 7th and 15th day of the experiment were also investigated. On the 15th day, blood samples for serum ALT, AST and FBG assay were collected through cardiac puncture under inhaled diethyl ether anaesthesia. Rat livers were also studied for histopathology. Results showed that treatment with APAP intraperitoneal injection induced significant ($P<0.001$) elevation in the serum levels of ALT and AST while inducing significant ($P<0.05$) decrease in the serum FBG. The hepatotoxicity was also corroborated by the histopathological lesion of lipid peroxidation characterized by diffuse ballooning degeneration with lymphocytic infiltration. Significant ($P<0.01$) weight loss and hypoglycaemia were also associated with APAP-induced hepatotoxicity. However, these alterations were attenuated in ASC pretreated rats dose dependently. Also, APAP-induced hypoglycaemia and weight loss were significantly ($P<0.01$, $P<0.001$) enhanced by ASC in dose related pattern. Thus, results of this study showed that 100 – 500 mg/kg/day was protective against APAP-induced hepatotoxicity, effect which could have been mediated via its inherent free radical scavenging and/or free radicals generation inhibiting activities.

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Keywords: *Acetaminophen-induced hepatotoxicity; Serum aminotransferases; Fasting blood glucose; Histopathology; Rats*

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INTRODUCTION

Acetaminophen (marketed as Panadol® in Great Britain, Tyelonol® in the US) is an effective, well-tolerated, widely used over-the-counter analgesic-antipyretic agent (Jalan *et al,* 2006), with more than 1 billion tablets sold annually in the United States alone (Nourjah and Wiley, 2002). Due to its high tolerance and its availability over-the-counter, overdose on acute or chronic use of the drug, causing fulminant hepatic is common, particularly, at daily doses greater than 4 g in adults (Ostapowicz *et al,* 2002). It is estimated that over 56,000 emergency visits and nearly 500 deaths occur in the US annually, resulting from acetaminophen toxicity, owing to either intentional or accidental overdoses (Nourjal and Wiley, 2002). However, these figures continue to rise (Bernal, 2003). In recent time, the safety of acetaminophen even at therapeutic doses has generated considerable debate (Jalan *et al,* 2006). Results of recent study by Watkins and co-workers as well as of other studies have reopened the controversies on the therapeutic safety of acetaminophen on long term continuous treatment (Kwan *et al,* 1995; Yin *et al,* 2001; Watkins *et al,* 2006). Acute liver injury has been reported in patients on therapeutic doses of acetaminophen (Critchley *et al,* 1986; Yin *et al,* 2001; Watkins *et al,* 2006). Acetaminophen-induced hepatic injury is reported to be mediated through an increased lipid peroxidation in hepatic tissues (Schnellman, 2001; Bessems and Vermeulen, 2001). However, therapeutic options for the treatment and the prophylaxis of acetaminophen-associated complications are limited (James *et al,* 2003). Thus, the search for chemoprophylactic agents for acetaminophen related hepatic complications becomes imperative.

Literature has shown the most abundant and effective antioxidant in the human body to be ascorbic acid (also known as vitamin C) (Frei *et al,* 1989). In view of the intrinsic antioxidant activity of this vitamin, the present study was designed to investigate the protective effect of 100 – 500 mg/kg of single, daily oral doses of the vitamin in high dose acetaminophen-treated Wistar rats for 14 days.

MATERIALS AND METHODS

Animals

All experimental procedures were conducted in strict compliance with the United States National Institutes of Health guidelines for Care and Use of Laboratory Animals in Biomedical Research (1985). Young adult Wistar rats, weighting 110 – 140 g were obtained from the Animal House of the College of Medicine Of the University of Lagos, Idi-Araba, Lagos, Nigeria, in the month of June, 2007. They were kept in polyethylene cages in the Animal House of the Lagos State University College of Medicine, Ikeja, Nigeria, and allowed to acclimatize for 14 days before use. The rats kept in controlled room temperature (24 ± 2 ºC) and humidity (65 - 80%) under a 12/12h light-dark cycle (light on 06:00h) with free access to standard rat chow (Livestock Feed, Ikeja, Lagos State, Nigeria) and tap water made available *ad libitum*.

Drugs

The drugs used in the experiment include ascorbic acid salt (Sigma Chemical Co. St. Louis, U.S.A.), acetaminophen injections (Juhel Paracetamol®, Lona Pharmaceuticals, New Delhi, India), normal saline (Unique Pharmaceuticals, Sango-Otta, Nigeria). All other reagents used in this study were of analytical grade.

Oral administration of drugs

Before the experiment began, the rats were fasted overnight but tap water was made available *ad libitum*. The rats were randomly divided into 5 groups of 6 rats per group. Groups I and II, which served as the negative and positive controls were orally administered 10 ml/kg of body/day of normal saline, respectively, except that the latter was administered 200 mg/kg of daily, single intraperitoneal acetaminophen 1 hour after oral administration of normal saline. Groups III - V rats were orally dosed with single, daily 100, 200 and 500 mg/kg of ascorbic acid, respectively, 1 hour before intraperitoneal injection of 200 mg/kg acetaminophen for 14 days.

Weekly body weight measurement

On days 1, 7, and 15 of the experiment, the rat

weights were measured using Mettler weighing balance (Mettler Toledo Type BD6000, Mettler-Toledo GmbH, Greifensee, Switzerland) and the difference in weight in reference to the initial weight per group was calculated on each occasion.

Blood Collection and Measurement of serum AST and ALT in treated rats

Following termination of the experiment on the day 14, the rats were fasted overnight for 14 hours. Blood samples for serum ALT and AST were obtained by cardiac puncture with 21G needle mounted on 5 ml syringe (Becton Dickinson S.A., Fraga, Spain) under diethyl ether anaesthesia (Sigma Chemical Co., St. Louis, U.S.A.). The blood samples obtained were collected into plain sample bottles and centrifuged at 3000 rev/min. for 30 minutes to separate sera. Serum AST, and ALT were all assayed using Randox Diagnostic kits (Randox Laboratories Ltd., Crumlin, U.K.). Transaminases activities were measured as kinetic reaction using IFCC method. The absorbance of reaction was determined at 546 nm by spectrophotometer.

Measurement of fasting blood glucose

Parts of the blood samples obtained from experimental rats by cardiac puncture were also collected into fluoride oxalate coated sample bottles. From these, sera were separated from the whole blood. The serum fasting blood glucose was determined by glucose oxidase method of Trinder (1969), using One Touch Basic Blood Glucose Monitoring System (LifeScan Inc., Milpitas, California, U.S.A.).

Histopathological studies of rat liver

After the animals were sacrificed, postmortem examination was performed and the rat livers were identified and carefully dissected out *en bloc* for histopathological examination. After rinsing the dissected liver in normal saline, sections were taken from each organ. The tissue was fixed in 10% formo-saline, dehydrated with 100% ethanol solution and embedded in paraffin. It was then processed into 4-5 µm thick sections stained with haematoxylin-eosin and observed under a photomicroscope (Model N - 400ME, CEL-TECH Diagnostics, Hamburg, Germany).

Statistical Analysis

Results were presented as mean ± S.D. for body weights while data for biochemical indices were expressed as mean ± S.E.M. of six observations. Statistical analysis was done using two-way analysis of variance followed by post-hoc test, Student-Newman-Keuls test on SYSTAT 10.6. Statistical significance was considered at $P<0.05$, $P<0.01$, and $P<0.001$.

RESULTS

Effect of repeated, intraperitoneal APAP injection on serum ALT and AST in normal and ASC pretreated rats for 14 days

Table 1 shows effect of repeated, intraperitoneal APAP administration and pre-treatment with oral 100 – 500 mg/kg ASC on the serum ALT and AST, for 14 days. As shown, the serum levels of these enzymes were significantly ($P<0.001$) elevated from normal values. However, oral ASC significantly ($P<0.05$, $P<0.01$, $P<0.001$) attenuated this elevation in the pretreated rats in dose related fashion.

Table 1: Effects of oral 100 - 500 mg/kg/day of ascorbic acid on liver function test in acetaminophen-induced hepatotoxic rat for 14 days

Group	Treatment	ALT (U/L)	AST (U/L)
I	10 ml/kg/i.p. normal saline	14.3 ± 1.5	16.7 ± 1.5
II	200 mg/kg/i.p. APAP	193.3 ± 16.5[a]	214.3 ± 18.1[a]
III	100 mg/kg/p.o. ASC + 200 mg/kg/i.p. APAP	115.0 ± 4.8[b]	145.0 ± 4.1[b]
IV	200 mg/kg/p.o. ASC + 200 mg/kg/i.p. APAP	51.7 ± 9.5[c]	82.0 ± 12.8[c]
V	500 mg/kg/p.o. ASC ± 200 mg/kg/i.p. APAP	21.7 ± 2.1[d]	33.3 ± 4.2[d]

[a]represents significant increase at $P<0.001$ when compared to group I values

[b, c, d] represent significant decrease at $P<0.05$, $P<0.01$, $P<0.001$, respectively, when compared to group II values

Histopathological effect of repeated APAP injection on the livers of normal and ASC pretreated rats for 14 days

Intraperitoneal injection of APAP for 14 days induced marked histopathological lesion which was characterized by diffuse ballooning degeneration, pyknotic nuclei of hepatocytes with lymphocytic infiltration of the hepatic porta triad (figure 2) when compared to the hepatocyte archictecture of normal liver (figure 1). However, the hepatocyte distortion was ameliorated in ASC pretreated livers, also, in dose related fashion. The most significant ameliorating effect was recorded for 500 mg/kg ASC-pretreated liver (figure 3).

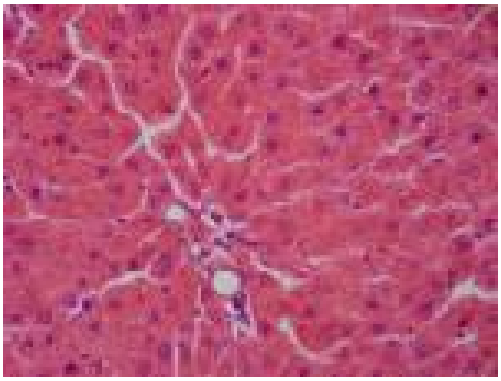

Figure 1.
A representative section of a normal rat liver showing the porta triad and normal hepatocytes

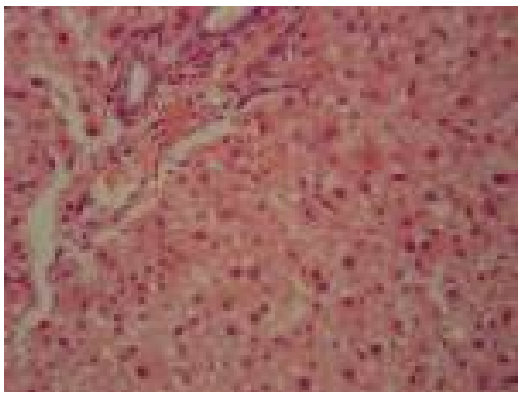

Figure 2.
A representative section of APAP-treated rat liver showing diffuse cytoplasmic vacuolation (ballooning degeneration), pyknotic nuclei of hepatocytes with lymphocytic infiltration of the hepatic porta triad

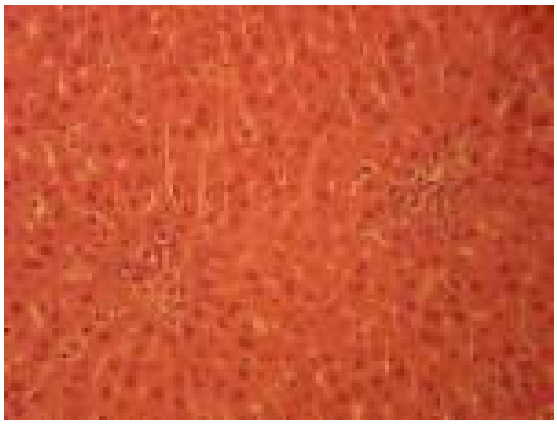

Figure 3.
A representative section of 500 mg/kg of ASC pretreated, APAP-treated rat liver showing the porta triad and normal hepatocytes

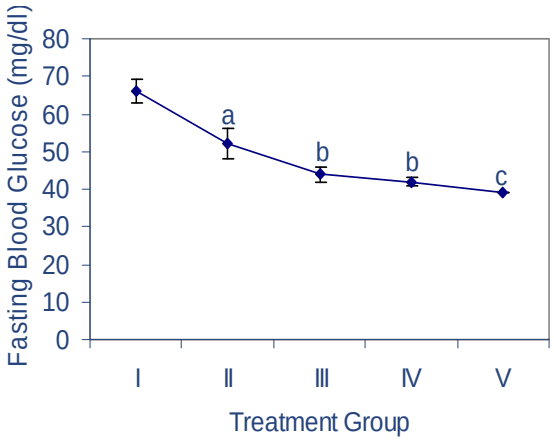

Figure 4.
Effect of graded oral doses of ASC on the fasting blood glucose concentration in acetaminophen hepatotoxic rats for 14 days

Effect of repeated, graded oral ASC doses on FBG in APAP hepatotoxic rats for 14 days

Figure 4 shows effect of repeated intraperitoneal injection of 200 mg/kg/day APAP on the serum fasting glucose in normal and ASC pretreated rats for 14 days. This dose induced significant ($P<0.001$) hypoglycaemia, effect that was significantly ($P<0.05$, $P<0.01$, $P<0.001$) further enhanced by graded oral ASC doses, dose dependently.

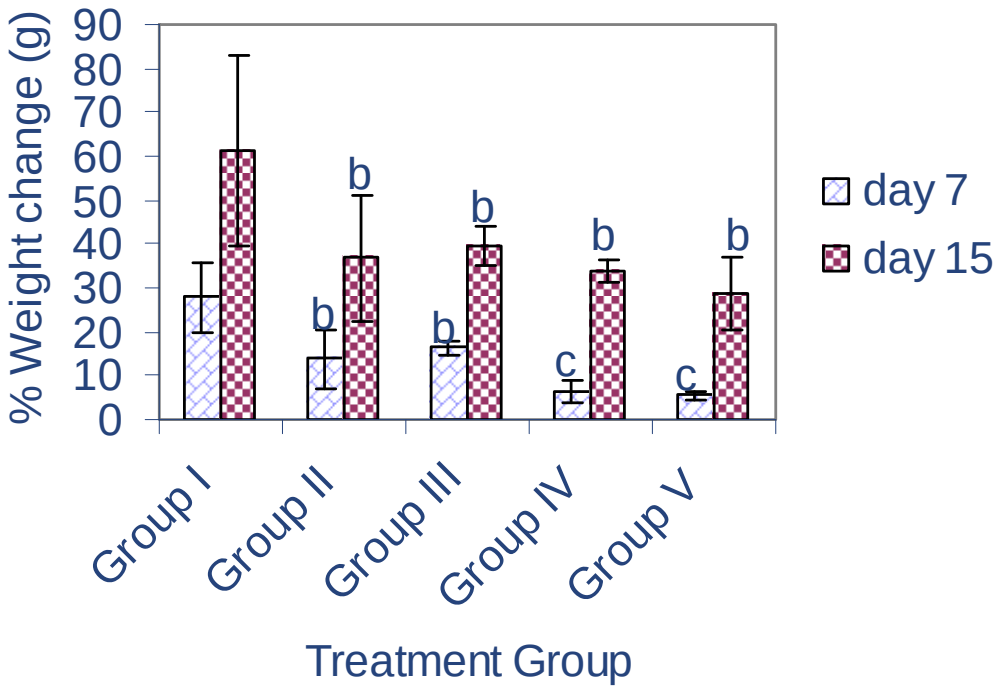

Figure 5. Effect of graded oral doses of ASC on the percentage weight changes in acetaminophen hepatotoxic rats on days 7 and 15

Effect of repeated APAP intraperitoneal injection and graded oral ASC pretreatment on the average body weight of rats

As shown in figure 5, single daily intraperitoneal injection of APAP for14 days, induced significant ($P<0.05$) weight loss in the treated rats. This was significantly ($P<0.01$, $P<0.001$) enhanced by oral ASC, also, in dose dependent fashion.

DISCUSSION

The non-steroidal analgesic-antipyretic drug, acetaminophen, is one of the safest over-the-counter drugs when used in recommended doses, but is capable of producing massive hepatic necrosis on acute overdose or chronic low dose use (Prescott *et al*, 1971; Wilkinson *et al*, 1977; Bonkovsky *et al*, 1994). However, different treatment strategies have been proposed to ameliorate or prevent acetaminophen-induced hepatotoxicity (Mitchell *et al*, 1974; Bessems and Vermeulen, 2001). In the present experimental animal study, acetaminophen-induced hepatic injury was induced by repeated intraperitoneal injection of 200 mg/kg of acetaminophen for 14 days. Mechanisms of acetaminophen hepatotoxicity have been extensively studied (Mitchell *et al*, 1974; Comporti *et al*, 1991). Excessive formation of a highly electrophilic and reactive intermediate metabolite, N-acetyl-para-benzoquinone-imine (NAPQI), which preferentially conjugates with hepatic glutathione, occurs when large doses of the drug are ingested (Lee, 1995). When the glutathione is exhaustively depleted, the NAPQI arylates essential nucleophilic macromolecules within the hepatocytes, forming stable acetaminophen-protein adducts, which represent the initial and irreversible step in the development of acetaminophen-induced hepatotoxicity (Comporti *et al*, 1991). In addition to NAPQI formation,

highly reactive free radicals are generated by oxidative reaction of cytochrome P450 (Farrell, 1994). These free radicals can also bind covalently to proteins and unsaturated fatty acids of cell membranes, resulting in lipid peroxidation, cellular membrane distruption, depressed mitochondrial function and consequent hepatocyte death (Farrell, 1994). However, literature has shown acetaminophen hepatotoxicity to be characterized by marked serum elevation of the liver enzymes (ALT and AST) (Rabinovitz *et al*, 1992; Schellman, 2001). ALT and AST are the most frequently utilized of hepatocellular injury and represent markers of hepatocellular markers (Boyde and Latner, 1961). Of these enzymes, ALT is localized primarily to the liver whereas AST is found in a wide variety of other tissues such as the cardiac and skeletal muscle, kidney, brain, etc. (Friedman *et al*, 1996). Thus, ALT is considered the most reliable marker of hepatocellular injury as diseases of skeletal and cardiac muscles, kidneys and the brain can equally elevate circulating levels of AST (Friedman *et al*, 1996). Results of the present study showed that intraperitoneal administration of 200 mg/kg/day of acetaminophen for 14 days reliably induced markedly significant ($p<0.001$) elevation of serum levels of AST and ALT in group II rats. However, these elevations were significantly ($p<0.01$) attenuated by vitamin C pre-treatment in dose related fashion. The most significant ($p<0.001$) ameliorating effect was recorded at 500 mg/kg/day of ascorbic acid. The recorded biochemical values were substantiated by the histopathological changes which were characterized by diffuse ballooning degeneration, pyknotic nuclei of hepatocytes with lymphocytic infiltration of the hepatic parenchyma (indicative and reflective of acute hepatocellular injury). These are suggestive of a high level of lipid peroxidation in group II rats. Again, these hepatic changes were ameliorated in group III – V rats in dose related pattern, the most significant of which was recorded in group V rats. Vitamin C, as an antioxidant agent, may have inhibited the chain reactions of acetaminophen-generated free radicals or scavenged the reactive free radicals before reaching their hepatic targets. Both animal (Odigie *et al*, 2007) and human (Idogun and Ajala, 2005) studies have shown ascorbic acid to be a potent antioxidant which mediates its antioxidant effect by scavenging free reactive oxygen species (ROS). Other studies have equally shown the protection of ascorbic acid and other vitamins in hepatic oxidative damage (Barja *et al*, 1994; Appenroth, 1997). Thus, results of the present study suggests vitamin C's ameliorating effects to be likely mediated via inhibition of free radicals generation and/or free radical scavenging activity.

The effect of graded oral doses of ASC on the fasting blood glucose in acetaminophen-induced hepatotoxic rats is also remarkable. Literature has shown metabolic complications of APAP overdose to include hypophosphataemia, (as a result of accompanying phosphaturia), metabolic acidosis, hypoglycaemia, among others (Jones *et al*, 1989; Makin *et al*, 1995). APAP-induced hypoglycaemia is proposed to be mediated via several mechanisms which include impaired glycogeneolysis, gluconeogenesis and hyperinsulinaemia (Record *et al*, 1975). As shown in figure 4, acetaminophen-induced hepatotoxicity was associated with significant ($P<0.05$) hypoglycaemia. Thus, this result is in accord with earlier documented reports. However, the hypoglycaemic effect of APAP was significantly ($P<0.05$, $P<0.01$, $P<0.001$) further enhanced by oral ASC in dose related fashion, most significant ($P<0.001$) effect of which was recorded for group V rats. Also, the progressive and dose related weight loss could be related to increased metabolic state associated with prolonged hypoglycaemic state. However, this assertion requires further evaluation. One mechanism by which a drug could induce hypoglycaemia and subsequent weight loss is by suppressing appetite. This was not the case as there was no change in the feeding pattern in the ASC-pretreated rats compared to group II rats that showed significant reduction in their feeding pattern.

In conclusion, the overall results showed that oral ASC ameliorates hepatotoxic effect of repeated high dose acetaminophen, effect which was possibly mediated via free radical scavenging and/or inhibition of free radical generation.

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